Vaccinia as a vector for tumor-directed gene therapy: Biodistribution of a thymidine kinase-deleted mutant

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Tumor-directed gene therapy, such as "suicide gene" therapy, requires high levels of gene expression in a high percentage of tumor cells *in vivo* to be effective. Current vector strategies have been ineffective in achieving these goals. This report introduces the attenuated (thymidine kinase (TK)-negative) replication-competent vaccinia virus (VV) as a potential vector for tumor-directed gene therapy by studying the biodistribution of VV in animal tumor models. A TK-deleted recombinant VV (Western Reserve strain) expressing luciferase on a synthetic promoter was constructed. Luciferase activity was measured *in vitro* after transduction of a variety of human and murine tumor cell lines and *in vivo* after intraperitoneal (i.p.) delivery in C57BL/6 mice with 7-day i.p. tumors (10^6 MC-38 cells). Three other *in vivo* tumor models were examined for tumor-specific gene expression after intravenous delivery of VV (human melanoma in nude mice, adenocarcinoma liver metastasis in immunocompetent mice, and subcutaneous sarcoma in the rat). In addition, a replication-incompetent vaccinia (1 μ g of psoralen and ultraviolet light, 365 nm, 4 minutes) was tested *in vitro* and *in vivo* and compared with active virus. Luciferase activity in i.p. tumors at 4 days after i.p. injection of VV was >7000-fold higher than lung, >3000-fold higher than liver, and >250-fold higher than ovary. In addition, intravenous injection of VV resulted in markedly higher tumor luciferase activity compared with any other organ in every model tested (up to 188,000-fold higher than liver and 77,000-fold higher than lung). Inactivation of the virus resulted in negligible gene expression *in vivo*. In summary, VV has a high transduction efficiency in tumor cells with high levels of gene expression. The results suggest a selective *in vivo* replication of TK-deleted VV in tumor cells. Replication competent, TK-deleted VV appears to be an ideal vector for testing the *in vivo* delivery of toxic genes to tumor cells. **Cancer Gene Therapy (2000) 7, 66–73**

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In vivo cancer gene therapy efforts have been disappointing. Inefficient gene delivery and expression, lack of specific tumor targeting, and immune clearance represent major obstacles that have yet to be overcome by current vectors.¹ The ideal tumor-targeted cancer gene therapy vector must transduce cancer cells with high efficiency, such that the majority of cells within a tumor express the gene of interest. Unlike gene therapy for congenital disorders of metabolism, this can be a transient expression system. However, it requires high levels of expression to assure tumor cell response and increase the chance of bystander killing. Most importantly, the vector must specifically target the tumor cells to provide a therapeutic advantage over normal cells.

Pox viruses have many characteristics that make them

ideal vectors in the setting of cancer gene therapy.² They have a high efficiency of transduction with high levels of gene expression. Unlike most vector systems, which rely entirely on the host transcription machinery, poxviridae encode for their own transcriptional enzymes. Gene expression occurs within the cytoplasm of the cell and is not dependent upon host cell replication. Synthetic pox promoters represent the strongest eukaryotic promoters available, leading to high levels of gene expression within the cell. Pox viruses also have the advantage of a large genome, which allows for recombination of ≤ 25 kilobases of DNA, and it is easy to grow high titers of virus.³ In addition, vaccinia virus (VV) has been used excessively in humans for smallpox vaccinations and has been shown to be safe.

Although much attention has been given the use of VVs as vaccines in the immunotherapy of cancer, few reports have addressed vaccinia as a vector for tumor-directed gene therapy. In addition to the advantages of this vector that have already mentioned, some groups have reported that VV selectively targets to tumor tissue *in vivo*.⁴ A thymidine kinase (TK)-deleted VV is highly attenuated in nondividing cells but is still able to replicate in transformed cells.⁵ This may lead to tumor-

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selective replication *in vivo*. In addition, as more becomes known about the mechanisms of VV binding and uptake into cells, we may find that there exists a tropism based on tumor cell surface receptors.

The purpose of this study was to evaluate VV (Western Reserve (WR) strain, TK mutant) as a vector for tumor-directed gene therapy by performing marker studies in animal tumor models. We have examined *in vitro* tumor cell transfection efficiency and *in vivo* expression of a marker gene in tumor and normal tissue after different routes of virus delivery in different tumor models. We have similarly studied a psoralen ultraviolet (UV) light-inactivated VV to compare its toxicity and efficiency with that of a replication-competent virus.

MATERIALS AND METHODS

Cell lines and cell culture

The human adenocarcinoma lines H 508 (CCL 253, American Type Culture Collection (ATCC), Manassas, Va), WiDR (CCL 218, ATCC) and HT-29 (HTB 38, ATCC), as well as the murine adenocarcinoma line CMT93 (derived from C57BL/6) were purchased from ATCC (CCL 223). MC-38 is a nonmetastatic methylcholanthrene-induced murine adenocarcinoma derived from C57BL/6 mice. MCA sarcoma is a methylcholanthrene-induced sarcoma derived from Fischer rats, originally established in our branch. P-mel is a human melanoma line established from a patient treated at the National Institutes of Health. All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) (Biofluids, Rockville, Md) supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, and 50 U/mL penicillin/streptomycin. HeLa S3 cells (CCL 2.2, ATCC) were used for the amplification of viral vectors. They were grown in spinner flasks (Belco Glass, Inc., Vineland, NJ) in DMEM containing 5% horse sera (Biofluids), 2 mM glutamine, and 50 U/mL penicillin/streptomycin. CV-1 cells (CCL 70, ATCC), which were used for recombination and standard plaque assays, were maintained in complete DMEM as described above.

β -galactosidase (β -gal) and luciferase expression vectors

VJS-6, an *Escherichia coli* *lacZ*-expressing VV under the control of the natural p7.5 early/late promoter, was kindly provided by B. Moss (Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Md). A new vaccinia recombination plasmid was constructed for recombination into the vaccinia TK gene as follows. The *Xba*I site of *pSC65* (provided by B. Moss) within the flanking region of the TK gene was abolished with Klenow and deoxynucleotide triphosphate filling. The plasmid was then amplified with primers to introduce a *BSSH II* site between the TK flanking region, allowing for the insertion of the multiple cloning site of pBluescript KS II⁺ (Stratagene, La Jolla, Calif).

To prevent possible antisense transcripts driven by the native vaccinia TK promoter, two primers were designed to encode an early termination signal (TTTTTNT) for all three possible open reading frames (5'-ATC GGA GCT CTT TTT ATC TGC GCG GTT AAC CGC CTT TTT ATC CAT and 5'-ATC GTC TAG ACT CCA CAA TAA AAA CAG ATC ACC TGA TGG ATA AAA). The primers were hybridized, and deoxynucleotide triphosphates and Klenow enzyme were added to the reaction mixture. After restriction with *Sac*I/*Xba*I,

Figure 1. VV shuttle plasmid encoding firefly luciferase. The expression cassette is flanked by the coding sequence of the VV TK gene (vTK-L, vTK-R). Xanthine-gpt from *E. coli*, driven by the vaccinia p7.5 early/late promoter, was employed to select for recombination events. The marker gene luciferase is driven by an optimized synthetic early/late promoter to maximize expression (Pse/L = AAGCTTAAAAATTGAAATTTTATTTTTTTTTTTTGGGAATATAAATAGCTCGA). A VV termination fragment in all possible coding frames prevents antisense transcripts from the vaccinia TK gene.

the termination signal was gel-isolated and ligated into the above plasmid. *Guanine phosphoribosyl transferase* (*gpt*) was cut out of *pBSgpt* (kindly provided by Dr. Richard Moyer, Department of Molecular Genetics and Microbiology, University of Florida, Gainesville, Fla) using *Xho*I and *Eco*RV and inserted into the multiple cloning site of the resulting plasmid *pCB022*. A promoter cassette consisting of a VV p7.5 early/late and a synthetic early/late promoter^{6,7} in sense and antisense direction was inserted immediately upstream so that the *gpt* gene was under the control of the p7.5 promoter. Finally, the luciferase gene was cut out of *pGEM-luc* (E1541, Promega, Madison, Wis) and inserted into *pCB025* to be driven by the vaccinia synthetic early/late promoter (Fig 1).

VV (WR strain) recombination was performed using CaCl₂ precipitation,⁸ and recombinant VV (recVV) was subsequently isolated by mycophenolic acid selection.

Immunohistochemistry and β -gal staining

For immunohistochemistry, whole organs were harvested and immediately fixed in formalin 10%. Paraffin embedding, sectioning, and subsequent staining using a polyclonal rabbit anti- β -gal antibody (UCB-A720/R6H, Accurate, Westbury, NY) were performed at Paragon Biotech (Baltimore, Md).

Organs for 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-Gal) staining were washed in phosphate-buffered saline (PBS) for 10 minutes to remove the rest of the blood. X-Gal solution was freshly prepared by adding 1.3 mM MgCl₂, 3 mM K₃Fe(CN)₆, 3 mM K₄Fe(CN)₆, 50 μ l of 10% Triton X-100, and X-Gal (5520UC, Life Technologies, Gaithersburg, Md) to PBS (10 mL). Organs were stained in X-Gal for 15 minutes without glutaraldehyde fixation.

Psoralen inactivation

For *in vitro* studies, 10^8 plaque-forming units (PFU) of VV were incubated in a six-well plate with 1 μ g of psoralen (4'-aminomethyl-trioxsalen; Calbiochem, La Jolla, Calif) in a total volume of 1 mL Hanks' balanced salt solution/0.1% bovine serum albumin for 10 minutes. The dish was irradiated with 365 nm of longwave UV (LWUV) in a Stratalinker 1800 (Stratagene) for 4 minutes.⁹ Higher PFUs for *in vivo* experiments were inactivated in the same fashion. The virus was assayed for remaining replication competent virus by standard plaque assay as described previously.⁸

Studies in vitro

A total of 5×10^5 CV-1 cells were plated in six-well plates and grown to confluence overnight. The cells were infected with vCB025 or VJS-6 at a multiplicity of infection (MOI) of 5 for 2 hours. Fresh media were added, and the plates were incubated until further evaluation. For measurement of luciferase activity, cells were washed twice with PBS and subsequently lysed with 500 μ L/well reporter lysis buffer (E4030, Promega). Samples were evaluated in a standard luciferase assay in a Turner Design Luminometer TD-20/20 (E2051, Promega). Due to the high sensitivity of the assay and to minimize loading errors, each sample was measured at least in duplicate and the average value was determined. To compare the level of gene expression, luciferase activity was normalized against protein concentration (23225, Pierce, Rockford, Ill) and expressed in relative light units (RLU) per microgram of protein. The VJS-6-infected cells demonstrated no measurable luciferase activity.

Studies in vivo

Peritoneal carcinomatosis. To establish peritoneal carcinomatosis, 10^6 MC-38 tumor cells in 4 cc of DMEM were injected intraperitoneally (i.p.) in C57BL/6 mice. On day 7 after tumor implantation, 10^9 PFU of VV were injected i.p. in a total volume of 2 mL. Animals were sacrificed for luciferase activity at various timepoints.

Hepatic metastases in the mouse. A total of 10^6 MC-38 tumor cells were injected intrasplenically in C57BL/6 mice on day 0, followed immediately by splenectomy. On day 7, 10^8 PFU of VV were injected intravenously (i.v.) (by tail vein), and animals were sacrificed for luciferase activity on day 11 (4 days after virus injection).

Subcutaneous (s.c.) sarcoma in the rat. A total of 10^6 rat MCA sarcoma cells were injected into the s.c. tissue of the flank of Fischer rats. On day 14, after the development of palpable tumor nodules, 10^8 PFU of VV were injected i.v. (by tail vein); tissue was collected 48 hours later for luciferase assays.

S.c. human melanoma in nude mice. A total of 1.5×10^6 P-mel tumor cells were injected into the s.c. tissue of the flank of athymic mice (C57BL/6) on day 0. On day 28, after the development of palpable tumors, 10^6 PFU of VV were injected i.v. (by tail vein). Animals were sacrificed 48 hours later for tissue luciferase assays.

On the day of sacrifice, the animals were euthanized and tumor and organs were harvested without delay, washed in PBS to remove remaining blood, and immediately homogenized for 30 seconds using a tabletop tissue homogenizer (FastPrep FP120 No. 6000-120, Bio 101/Savant, La Jolla, Calif). For each sample, supernatant was collected and stored at -70°C until the completion of each experiment and collective measurement of all samples for luciferase activity. Lucif-

Table 1. VV Expresses Luciferase Marker Gene Efficiently in a Variety of Different Cell Lines *In Vitro**

Cell lines	Luciferase activity (RLU/min/ μ g protein)
H508	8587
WiDR	8831
CMT93	1936
HT-29	10739
MC-38	10800

*Duplicate wells of confluent cells (10^6 cells/well in a six-well plate) were infected at an MOI of 5 with either vCB025, a firefly luciferase-expressing VV, or control virus VJS-6, which expresses β -gal. Luciferase activity was measured after 48 hours and normalized against the protein concentrations of the lysate. VJS-6 control virus-treated cells demonstrated no measurable luciferase activity.

erase activity was measured for each sample, and the mean plus SD value for each group was calculated. Control VJS-6-injected animals demonstrated no measurable luciferase activity.

RESULTS

Luciferase expression in different cell lines

Five different cell lines were grown to confluence in six-well plates. The cell number per well was established in parallel to account for differences in growth pattern and morphology. The cell lines were infected with vCB025 at an MOI of 5 for 2 days. High luciferase activity could be found in all human adenocarcinoma lines as well as in the two murine tumor lines demonstrating a broad infectivity range for the VV. There was consistent variability, however, with the CMT93 (mouse adenocarcinoma) having the least levels of expression, and HT-29 (human adenocarcinoma) and MC-38 (mouse adenocarcinoma) having the highest levels (Table 1). Cell lines infected with VJS-6 at an MOI of 5 demonstrate that 100% of cells express β -gal at 24 hours (data not shown). VJS-6-infected cells demonstrated no measurable luciferase activity.

Luciferase expression in vivo

The maximum tolerated dose (MTD) of live VV was determined for i.v. and i.p. administration as 10^8 PFU and 10^9 PFU, respectively, in immunocompetent C57BL/6 mice. In a peritoneal carcinomatosis model, C57BL/6 mice (five mice per group) were injected i.p. with vCB025 on day 7 after tumor implantation (10^9 PFU). Organs were harvested for luciferase gene expression after 2 days. VJS-6-injected control animals demonstrated no measurable luciferase activity.

Virus administration directly into the peritoneal cavity led to markedly higher luciferase activity in the tumor compared with all other tissues assayed. The tumor luciferase activity was 16,354-fold higher than the lung, 23,332-fold higher than the liver, and 673-fold higher than the heart. Minimal luciferase activity was measured in some of the intra-abdominal tissues, such as the

Table 2. Luciferase Gene Expression is Selective for Tumor Samples*

Luciferase activity (virus delivery)	(RLU/min/mg protein)								
	Tumor	Liver	Spleen	Kidney	Uterus/ovaries	Peritoneum	Muscle	Lungs	Heart
i.p. (10 ⁹)	174990	7.5	63.7	9.1	694	42.7	0	10.7	260

*C57BL/6N mice ($n = 5$) were challenged at day 7 after tumor implantation (10^6 cells, i.p. MC-38 adenocarcinoma) with the MTD of replication-competent VV vCB025. After 2 days, the animals were sacrificed, the organs were harvested and homogenized, and luciferase activity was determined. RLU were normalized to the protein concentration. VJS-6-injected control animals demonstrated no measurable luciferase activity.

peritoneal lining and spleen. No luciferase activity was identified in the brain. Luciferase activity in the ovary was consistently found to be the highest of all organs (other than tumor) throughout every experiment. Luciferase activity, however, was ≥ 250 -fold higher in tumor samples than ovary (Table 2). These findings were confirmed by immunohistochemistry and whole organ X-Gal staining, which showed selective marker gene expression in the tumors but not in adjacent organs (Fig 2). The bowel did not express any marker genes when stripped of the peritoneal lining, which is prone to tumor implantation and hence "contamination" with luciferase activity.

Table 3 summarizes the data from the other tumor models. In the murine model of hepatic metastases, the tumor diffusely replaces the liver by the time of sacrifice. Tumor luciferase activity was 7666-fold greater than the lung and 3172-fold greater than the ovary (normal liver could not be separated from the tumor and therefore was not assayed). The s.c. sarcoma in the rat also showed selective gene expression in the tumor after i.v. injection of virus. Overall, the luciferase activity was lower in this model compared with the murine model, despite a larger dose of virus (10^8 PFU in rats versus 10^6 PFU in mice). Rats were sacrificed only 2 days after virus injection, and it may take more time than this to reach maximum levels of luciferase activity. Nevertheless, rat tumor luciferase activity was $>188,000$ -fold higher than liver and $>77,000$ -fold higher than lung activity. Interestingly, even the s.c. human melanoma model in athymic mice showed markedly increased luciferase activity in tumors compared with all other organs. Relative to the tumor, however, the other organs showed an increase in luciferase activity compared with other models. The tumor had 2595-fold increased activity compared with the lung, but only 7-fold increased activity compared with the ovary.

Duration of gene expression in vivo

The duration of gene expression *in vivo* was established in immunocompetent mice by i.p. injecting tumor-bearing animals on day 7 after tumor implantation with the MTD (10^9 PFU) of vCB025 into the peritoneal cavity. Animals were sacrificed for luciferase assays on days 2, 4, 6, 7, 8, and 10 (five animals per group). All organs showed maximum luciferase activity at day 4 after virus delivery. Luciferase activity remained high until day 6 and declined on day 7 (Fig 3). Only tumor tissue

demonstrated gene expression after day 7. The tumor luciferase activity on days 4 and 6 was >1800 -fold higher than the level of the uterus/ovary and >2800 times higher than luciferase activity within the liver, the second highest other organ.

Psoralen/UV-inactivated VV

To abolish viral replication, vCB025 and VJS-6 were psoralen/LWUV-inactivated as described in *Materials and Methods*. Viral replication and the cytopathic effect (CPE) were completely abolished with 10^9 PFU/mL treated with $1 \mu\text{g}$ psoralen/mL and LWUV for 4 minutes (Fig 4). For *in vivo* experiments, 10^{10} PFU/mL virus was treated with $1 \mu\text{g}$ psoralen/mL and LWUV for 4 minutes, resulting in 5×10^6 PFU replication-competent viral particles and inactivation of $>99.9\%$ of the total virus population.

To compare the duration of *in vitro* luciferase activity after infection with replication-competent versus psoralen/LWUV-inactivated virus, CV-1 cells were grown to confluence overnight and infected with vCB025 at an MOI of 5. Cells were harvested at different timepoints and assayed for luciferase activity. Maximal luciferase activity for both groups was reached at 24 hours. However, there was an almost 600-fold higher peak of luciferase activity in cells infected with the active versus inactivated virus (Fig 5). Due to the cytotoxicity of active VV, luciferase activity declined in parallel with the number of living cells within the dish. The luciferase activity in the inactivated virus surpassed the active virus on days 7–10, as more cells remained viable. Although peak gene expression is reduced with inactivation, the elimination of viral replication and the CPE have the potential advantage of decreased systemic toxicity, allowing delivery of higher viral titers.

To compare the *in vivo* luciferase gene expression of replication-competent and psoralen-inactivated VV, animals bearing tumors for 7 days were injected i.p. with 10^9 PFU of active or inactivated vCB025. The luciferase activity in tumor samples was determined every 2 days for 10 days after virus injection (Table 4). Luciferase activity in the tumor was again detectable at 2 days in the active virus group, reached a peak at 4 days after tumor implantation, and started to decline after 6 days. This finding was highly reproducible and correlated with immunohistochemistry. Psoralen-inactivated virus groups (10^9 PFU, $1 \mu\text{g}$ of psoralen, LWUV for 4

Figure 2. X-Gal staining of organs and immunohistochemistry. C57BL/6N mice were injected i.p. with syngeneic MC-38 adenocarcinoma cells (10^6). At day 7 after tumor implantation, 10^9 PFU of VJS-6 were injected i.p., and the animals were sacrificed after 5 days. Organs were harvested, washed in PBS, and either stained for β -gal or fixed in 10% formalin for immunohistochemistry. Whole organ X-Gal staining was performed for 15 minutes. **A:** Omental tumor implant: the entire tumor surface is convincingly stained by X-Gal. **B:** Immunohistochemistry of tumor tissue reveals staining deep within the tumor, suggestive of i.t. viral spread and amplification. **C:** Murine spleen with adjacent tumor and fatty tissue. Although tumor tissue stains heavily blue for β -gal marker gene activity, adjacent fat tissue and spleen do not. **D:** Immunohistochemistry confirms selective marker gene expression within tumor tissue but not within splenic parenchyma. The staining pattern shows penetration of VJS-6 deep into tumor layers. **E:** Whole organ staining of the entire gastrointestinal tract confirms selective X-Gal staining of tumor nodules. Bowel loops and stomach do not display β -gal activity. **F:** Immunohistochemistry of tumor deposit (positive for marker gene) next to bowel section (negative for marker gene).

minutes) did not show luciferase above background at the majority of timepoints.

DISCUSSION

Tumor-directed gene therapy efforts have been hampered by the lack of suitable delivery vectors. Tumor-specific vector targeting and replication could theoretically lead to direct lysis of tumor cells as well as enhance local therapeutic gene expression, leading to an antitumor effect. Pox viruses have known characteristics that may be ideal for tumor-directed gene therapy. As cyto-

plasmic viruses encoding their own transcriptional enzymes, they have high transduction efficiencies with strong promoter systems.¹⁰ Although *in vivo* gene expression is transient because of rapid immune clearance, this may be sufficient time for the activity of therapeutic genes. In addition to these known characteristics, we have demonstrated here selective expression of a marker gene within tumor tissue *in vivo* after the administration of VV.

Current vector strategies for tumor-directed gene therapy are predominately based on replication-incompetent retroviruses, adenoviruses, and nonviral delivery systems. More recently, herpes virus has been explored

Table 3. Luciferase Activity in the Highest Organs After i.v. Delivery (by Tail Vein) of 10^6 PFU VAC-LUC in Different Tumor Models*

Tumor model	Luciferase activity (RLU/min/mg protein)			
	Tumor	Ovary	Liver	Lung
Adenocarcinoma liver metastases in immunocompetent mice	4,600,000	1,450	—†	600
S.c. sarcoma in the rat	4,337	0.74	0.023	0.056
Human melanoma in athymic mice	558,000	78,000	215	963

*n = 5/group. Control VJS-6-injected animals demonstrated no measurable luciferase activity.
†Could not adequately separate tumor from normal liver in this model.

Figure 3. *In vivo* luciferase activity plateaus between days 4 and 6 after recVV challenge. Tumor-bearing animals (peritoneal MC-38) were treated at day 7 after tumor implantation with 10^9 PFU of vCB025 i.p. Five animals per group were sacrificed every 2 days, and organs were harvested and assayed for luciferase activity as described above. Gene expression was normalized to sample protein concentration. Control VJS-6-injected animals demonstrated no measurable luciferase activity.

as a constitutive replication-competent virus in tumor cells after the deletion of virulence genes.¹¹ It has properties similar to vaccinia but has the disadvantage of being a human pathogen and having more limited tropism. VV (WR strain, TK mutant) has theoretical advantages over these other systems for tumor-directed gene therapy. We have shown that vaccinia efficiently infects and expresses marker genes in a variety of cancer cell lines across different species. An MOI of 5 infects 100% of cells *in vitro*. This compares favorably with the efficiency of adenovirus in which an MOI of ≤ 100 is required for 100% of cells to express the transgene. When tested *in vivo*, VV also specifically expresses a marker gene in tumor tissue at high levels compared with all other tissues. Luciferase activity was >650 times higher in the tumor than the liver, lungs, and heart at day 2 postinfection and >2800 times higher on days 4 and 6 postinfection. This allows for a good therapeutic ratio for toxic gene expression. Only the ovary was shown to also specifically express vaccinia marker genes compared

with other tissues, but at much lower levels than in tumor tissue. Although this has theoretical disadvantages in terms of germline transmission of mutations, vaccinia does not insert into the genome and therefore should be safe. In addition, the loss of the ovaries in the case of toxic gene expression is not as devastating as with other organs, such as the lung, liver, or brain.

Although we have not studied the mechanism for this specific targeting, we hypothesize it is related to tumor permissiveness to viral replication. Although the deletion of TK within the viral genome leads to marked attenuation *in vivo*, it does not affect plaque formation in transformed cell lines. Metabolically active cells such as tumor cells have a higher pool of functional nucleotides that compensate for the loss of viral TK. The mechanism of vaccinia infectivity is not fully understood, and therefore it is not possible to study tumor cell expression of specific receptors to explain tropism. Recent data suggest that a major factor in viral infection is the binding of the viral protein A27L to cell surface heparin sulfate

Figure 4. β -gal expression of psoralen/LWUV-inactivated VV and active VJS-6. To test the influence of psoralen inactivation on gene expression, confluent CV-1 cells in six-well plates were infected at an MOI of 5 with VJS-6 (inactivation: psoralen $1 \mu\text{g}/10^8$ PFU, LWUV for 4 minutes) and tested for the CPE and for X-Gal staining. **A:** Virus inactivation abrogates the CPE almost completely. Concomitantly, there is a reduction of β -gal transcription due to random intercalation of psoralen. **B:** 100% cell staining is achieved at an MOI of 5 using active, replication-competent VJS-6. The CPE is clearly visible.

Figure 5. Luciferase expression of replication-competent and psoralen-inactivated vCB025 *in vitro*. CV-1 cells were grown to confluence in six-well plates overnight (10^6 cells/well). The cells were infected with either active or replication-incompetent vCB025 at an MOI of 5. Inactivation was achieved by the addition of $1\text{ }\mu\text{g}$ of psoralen to 10^8 PFU in 1 mL Hanks' balanced salt solution/0.1% bovine serum albumin and radiation with LWUV (365 nm) for 4 minutes. Wells were evaluated in duplicate.

proteoglycans, and that viral uptake can be inhibited by heparin.¹² There is some evidence to suggest that heparin sulfate is made more accessible in the tumor environment during digestion of the extracellular matrix.¹³ These factors should be applicable to the human host. Humans are not natural hosts for VV, but human tumors are able to be infected with vaccinia both *in vitro* and *in vivo*.

Local immune suppression within tumor tissue may also enhance the magnitude and duration of marker gene expression within tumors compared with other organs. Tumor cells can acquire immune resistance by interfering with the presentation of foreign antigens on major histocompatibility complexes.¹⁴ Failure to present endogenous viral proteins¹⁵ could result in delayed elimination of virus-infected tumor cells.¹⁶ This may explain why there is more relative tumor luciferase activity compared with the ovary in immune-competent mice, whereas in athymic mice the ratio of tumor to ovary activity is only 7. If local immune suppression in the tumor leads to increased relative gene expression in the tumor compared with other organs in immunocompetent animals, then this effect might be lost in immunosuppressed animals. Alternatively, because the tumor model is different, it may be that this human melanoma line is simply less permissive for vaccinia infection and replication than the other tumors tested.

Others have shown the ability of vaccinia to selectively replicate within tumor tissue *in vivo* after intratumoral (i.t.) inoculation of recVV in an immunocompetent host.¹⁷ In that report, i.t. viral titers increased by 10,000-fold over a 5-day period. By day 8, however, only minimal titers of vaccinia could be demonstrated in tumor lysates. Our *in vivo* results extend these observations using a marker of gene expression instead of viral recovery. Our impression is that viral replication within tumor tissue in our models *in vivo* leads to a 1100-fold increase in luciferase activity from day 2 to day 4. For an enzyme/prodrug therapeutic gene, it would be advantageous to begin prodrug therapy on day 4 after viral inoculation in an immunocompetent host. Although the psoralen/UV inactivation of vaccinia allows for higher safe vector delivery *in vivo*, there was a negligible level of gene expression in the tumor in contrast to replication-competent virus. The small difference in MTD does not compensate for this large difference in expression. It is our conclusion that a replication-competent VV has better therapeutic potential than an inactive virus.

A major disadvantage of vaccinia in the context of tumor-directed gene therapy is the immunogenicity of the vector and the fact that the majority of cancer patients have been immunized against vaccinia as part of the small pox eradication project. Although this may be a limiting factor to the vector, it is also possible that it

Table 4. *In Vivo* Luciferase Activity in i.p. Tumors (MC-38) with Active and Psoralen-Inactivated vCB025*

Virus	Luciferase activity (RLU/min/mg protein)				
	Day 2	Day 4	Day 6	Day 8	Day 10
Active vCB025 (10^9 PFU)	86.6	2446	1826	1.6	0
Psoralen-inactivated vCB025 (10^9 PFU)	0.3	9	0.2	4.2	0

*vCB025 was psoralen-inactivated as described previously ($1\text{ }\mu\text{g}$ psoralen of LWUV at 365 nm for 4 minutes). At day 7 after tumor implantation C57BL/6N mice were injected i.p. with 10^9 PFU of either active or psoralen-inactivated vCB025. Four animals per group were sacrificed every 2 days, and organs were harvested and assayed for luciferase activity as described previously. Gene expression was normalized to sample protein concentration.

can be used to its advantage. Data suggest that the *in vivo* bystander effect of the TK enzyme/prodrug system is dependent upon the immune response of the host to the vector.¹⁸ An immunogenic vector such as vaccinia, which destroys a tumor with a toxic gene, may lead to a markedly immunogenic *in vivo* tumor oncolysate that can improve the overall response and potentially eliminate microscopic metastatic deposits. Although pox viruses are being explored as vaccines against cancer, the trend is to move away from replication-competent viruses and to express tumor-associated antigens in cells other than tumors (antigen-presenting cells). The combination of the toxic destruction of tumor cells using a suicide gene and the immunogenicity of vaccinia may lead to significant responses. This strategy requires a window of replication and gene expression. We have observed that in patients who have been immunized against vaccinia in the distant past (>10 years), reimmunization with vaccinia leads to transient viral replication and pox formation in the skin (our unpublished observations). This finding suggests that at least transient replication and gene expression are possible in immunized patients.

In summary, VV as a member of the poxviridae could be a useful tool for tumor-directed gene therapy. The tumor-selective gene expression and its strong synthetic promoter systems are unique features that could be exploited for tumor-directed gene therapy efforts. The duration of gene expression in immunocompetent animals is short (<10 days), so any therapeutic genes must be potent, with a strong bystander effect. Although this remarkable targeting ability may not translate to humans, the murine model will allow the study of this system to help determine whether a tumor-specific replicating system could ever have therapeutic relevance in humans. Currently, we are investigating several enzyme/prodrug systems in recVVs both *in vitro* and *in vivo*.

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